Immunity to infection

Clinical

Research Article

Eosinophilia and reduced STAT3 signaling affect neutrophil cell death in autosomal-dominant Hyper-IgE syndrome

Susan Farmand[1,2,6] (iD), Bernhard Kremer[3], Monika Häffner[3],
Katrin Pütsep[2], Peter Bergman[1,4,5], Mikael Sundin[6,7],
Henrike Ritterbusch[3,8], Maximilian Seidl[3,9], Marie Follo[10],
Philipp Henneke*[3,8] and Birgitta Henriques-Normark*[1,2]

[1] Department of Clinical Microbiology, Karolinska University Hospital, Solna, Sweden
[2] Department of Microbiology, Tumor and Cell Biology, Karolinska Institutet, Stockholm, Sweden
[3] Center for Chronic Immunodeficiency (CCI), Medical Center, Faculty of Medicine, University of Freiburg, Freiburg, Germany
[4] Department of Laboratory Medicine, Clinical Microbiology, Karolinska Institutet, Stockholm, Sweden
[5] Immunodeficiency Unit, Infectious Disease Clinic, Karolinska University Hospital, Huddinge, Sweden
[6] Section of Hematology, Immunology and HSCT, Astrid Lindgren Children's Hospital, Karolinska University Hospital, Stockholm, Sweden
[7] Department of Clinical Science, Intervention and Technology, Karolinska Institutet, Stockholm, Sweden
[8] Center for Pediatrics and Adolescent Medicine, Medical Center, Faculty of Medicine, University of Freiburg, Freiburg, Germany
[9] Institute of Pathology, Medical Center, Faculty of Medicine, University of Freiburg, Freiburg, Germany
[10] Lighthouse Core Facility, Department of Medicine I, Medical Center, Faculty of Medicine, University of Freiburg, Freiburg, Germany

The autosomal-dominant hyper-IgE syndrome (HIES), caused by mutations in STAT3, is a rare primary immunodeficiency that predisposes to mucocutaneous candidiasis and staphylococcal skin and lung infections. This infection phenotype is suggestive of defects in neutrophils, but data on neutrophil functions in HIES are inconsistent. This study was undertaken to functionally characterize neutrophils in STAT3-deficient HIES patients and to analyze whether the patients' eosinophilia affects the neutrophil phenotype in S. aureus infection. Neutrophil functions and cell death kinetics were studied in eight STAT3-deficient patients. Moreover, the response of STAT3-deficient neutrophils to S. aureus and the impact of autologous eosinophils on pathogen-induced cell death were analyzed. No

Correspondence: Dr. Susan Farmand
e-mail: susan.farmand@ki.se

*These authors contributed equally to this work.

specific aberrations in neutrophil functions were detected within this cohort. However, the half-life of STAT3-deficient neutrophils ex vivo was reduced, which was partially attributable to the presence of eosinophils. Increased *S. aureus*-induced cell lysis, dependent on the staphylococcal virulence controlling accessory gene regulator (*agr*)-locus, was observed in STAT3-deficient neutrophils and upon addition of eosinophils. Accelerated neutrophil cell death kinetics may underlie the reported variability in neutrophil function testing in HIES. Increased *S. aureus*-induced lysis of STAT3-deficient neutrophils might affect pathogen control and contribute to tissue destruction during staphylococcal infections in HIES.

Keywords: HIES · STAT3 · neutrophils · eosinophils · *S. aureus*

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Introduction

The autosomal-dominant hyper-IgE syndrome (HIES) is a rare primary immunodeficiency caused by dominant-negative mutations in STAT3 [1, 2]. Associated multisystem features include eczema, delayed shedding of primary teeth, bone fractures, typical facial characteristics, craniosynostosis, and vascular abnormalities. Classical laboratory findings are increased serum IgE levels, low percentage of T_H17 cells, reduced memory B cells and blood eosinophilia [3].

STAT3-deficient HIES patients are particularly susceptible to mucocutaneous candidiasis and recurrent skin and respiratory infections with a rather narrow spectrum of pathogens, in particular *Staphylococcus aureus* (*S. aureus*), *Streptococcus pneumoniae* (*S. pneumoniae*), and *Haemophilus influenzae* (*H. influenzae*) [3]. Postinfectious pneumatoceles are pathognomonic, but patients also frequently develop bronchiectasis. These lung complications facilitate secondary opportunistic infections and have a major impact on morbidity and mortality in HIES [4]. Therefore, it seems essential to understand both the particular susceptibility to infection as well as the associated increased tissue destruction in HIES.

A major immunological hallmark of STAT3-deficient HIES is the lack of IL-17–producing T_H cells (T_H17) [5, 6]. T_H17 cytokines augment the production of chemokines and antimicrobial peptides, particularly from keratinocytes and bronchial epithelial cells. Thus, the lack of T_H17 may lead to deficient neutrophil recruitment and reduced antimicrobial killing in local infections, thereby offering an explanation for the observed susceptibility to mucocutaneous and tissue infections [7]. Whereas T_H17 immunity has been closely linked to mucocutaneous candidiasis, invasive lung infections with *S. aureus*, like in HIES, are not typical for other primary immunodeficiencies with reduced IL-17 signaling [8]. Furthermore, it was recently shown that STAT3-deficient patients with somatic mosaicism and a normal T_H17 response may still develop mucocutaneous candidiasis as well as pneumonia [9]. These observations imply that the lack of T_H17 alone is not sufficient to explain the specific susceptibility to staphylococcal lung infections in STAT3-deficient HIES. Defects in neutrophil function have been suggested to underlie the particular susceptibility to staphylococcal abscesses and lung infections and candidiasis [10–12]. In particular, altered chemotaxis has been reported repeatedly [11–15]. However, published data are inconsistent and variations even within the same patient have been observed [14–18]. In a recent study, STAT3-deficient neutrophils were found to migrate into blisters, indicating normal chemotaxis in HIES in vivo [9]. Although there might be temporary variations in the chemotactic capacity of STAT3-deficient neutrophils [13], the heterogeneity of findings, and the lack of correlation with *S. aureus* infections or disease severity in individual patients [15] challenges a direct causal relationship between impaired neutrophil chemotaxis and the clinical phenotype in HIES.

Overall, the discrepant data on neutrophil function in autosomal-dominant HIES is puzzling. Studies performed prior to the discovery of STAT3 deficiency as the genetic cause of HIES (2007) may be confounded by inclusion of patients with other causes of hyper-IgE syndrome. Another explanation for the variable results may be methodological differences in neutrophil function analysis. Density gradient centrifugation, the most commonly employed procedure for neutrophil isolation, leads to a minor contamination with eosinophils due to overlapping densities and thus to a mixed granulocyte preparation [19]. As eosinophil numbers are very low in healthy controls, their presence in these granulocyte preparations is usually considered negligible. However, the influence of increased eosinophil numbers in HIES patients has not been assessed. As eosinophils contain an arsenal of toxic substances that are released during incubation with *S. aureus* [20, 21], it is essential to understand their contribution to the clinical phenotype and functional neutrophil testing in HIES.

Here, we report an enhanced in vitro neutrophil cell death in STAT3-deficient HIES patients as compared to healthy controls. Our data suggest that both the presence of eosinophils and reduced STAT3 signaling affect neutrophil cell death kinetics and are associated with increased *S. aureus*-induced cell death.

Results

Variable clinical severity despite similar laboratory findings in STAT3-deficient HIES patients

The study cohort comprised six male and two female STAT3-deficient HIES patients between two and thirty years of age at the time of study inclusion (Table 1). Clinical laboratory findings retrieved from the patient charts showed typical immunological alterations found in STAT3-deficient HIES patients [3], such as elevated IgE levels, low or absent T_H17 cells/IL-17 levels, reduced percentages of marginal zone B cells and/or class-switched memory B cells (Table 1). Blood neutrophil numbers were normal in six patients, but were occasionally below normal limits in HIES #1 and in HIES #5. Eosinophilia, i.e. elevated eosinophil counts in blood (> 500 cells/µL), was documented in seven patients (Table 1). The investigated patients consistently showed relatively elevated eosinophil numbers in granulocyte preparations, including the patient with normal absolute eosinophil counts in whole blood. An exception was patient #8, who had a very high percentage of eosinophils in the granulocyte fraction (40%) during the initial analysis, but not at a subsequent analysis (2.5%).

The severity of clinical symptoms was highly variable between the patients, even in those with identical mutations (Table 2). All patients had a history of fungal infections. *S. aureus* was isolated from various body sites in seven of eight patients. Available clinical histology results revealed presence of neutrophils in abscesses of three patients and in the bronchoalveolar lavage (BAL) fluid of two patients. Furthermore, eosinophils were present in one BAL fluid sample and in three histological samples (Table 2, Supporting Information Fig. 1).

The patients received symptomatic treatment including antimicrobial prophylaxis, abscess drainage, and IgG-substitution when needed, but also lung surgery in three patients (data not shown).

Basic neutrophil functions were not altered in STAT3-deficient HIES patients

Several neutrophil functions were tested (Table 3). Due to limitations in sample size and availability not all tests could be performed for every patient. Reactive oxygen species (ROS)-release, chemotaxis, and adhesion were normal in the patients tested. Early whole blood and neutrophil-mediated staphylococcal killing was similar between investigated patients and controls. Neutrophils from patients released neutrophil extracellular traps (NETs) upon phorbol 12-myristate 13-acetate (PMA) stimulation (Supplement Movie 1–2, Supporting Information Figs. 2-4). In summary, STAT3-deficient HIES patients in our cohort did not show substantial alterations in basic neutrophil functions.

Cell death kinetics were accelerated in neutrophils from STAT3-deficient HIES patients

Quantification of cell death by flow cytometry (Fig. 1A–C) revealed accelerated cell death of STAT3-deficient neutrophils compared to concomitantly analyzed control cells (Fig. 1D). In particular, increased early apoptosis (intact cell membrane) (Fig. 1E) was found at four and nine hours after cell isolation and enhanced late apoptosis/necrosis (permeabilized cell membrane) (Fig. 1F) was evident at nine and fourteen hours. Interindividual differences were observed in both patients and controls. The percentage of eosinophils at the time of initial measurement, i.e. four hours after cell isolation, correlated strongly with the percentage of late apoptotic/necrotic neutrophils at four hours ($r = 0.92$; $p = 0.0002$) (Supporting Information Fig. 5). A less significant correlation ($r = 0.65$; $p = 0.0418$) was observed with the percentage of early apoptotic neutrophils at four hours. There was, however, no correlation with the percentage of eosinophils at later time points for any of the cell death categories. Cytokine stimulation with IL-8 increased cell viability throughout the observation time (Fig. 1G, Supporting Information Fig. 6). Still, a substantial difference in cell death remained discernible between patients and controls for both early apoptosis (Fig. 1H) and late apoptosis/necrosis (Fig. 1I) at nine and fourteen hours. Increased cell viability and reduced early apoptosis in combination with maintained differences in cell death between patient and control cells were also found for a limited number of samples treated with G-CSF and GM-CSF (Supporting Information Fig. 7). The enhanced cell death kinetics in HIES patients compared to controls were also observed during visual comparison of granulocyte suspensions by microscopy (Supporting Information Fig. 2C and D, Supporting Information Fig. 8) and during live cell imaging (Supplement Movie 3–6).

S. aureus-induced cell death was increased in STAT3 deficiency and upon addition of eosinophils

S. aureus is a major pathogen in STAT3-deficient HIES. We therefore analyzed the impact of *S. aureus* on neutrophil cell death in vitro. Both granulocyte preparations and highly purified neutrophils from STAT3-deficient patients consistently showed increased spontaneous and *S. aureus*-induced lytic cell death as compared to concomitantly analyzed control cells (Fig. 2A–D). When purified neutrophils were supplemented with 20% eosinophils *S. aureus*-induced cell death increased, both in cells from patients and controls (Fig. 2E). Due to sample size limitations the data shown in Figure 2 could not be pooled for statistics. However, to verify the effect of STAT3 inhibition and eosinophil supplementation on lactate dehydrogenase (LDH) release, the experiments were repeated with cells from healthy donors employing a chemical STAT3 inhibitor (ST18).

In contrast to cells isolated from HIES patients, chemical STAT3 inhibition of control granulocytes or purified neutrophils alone did not substantially affect spontaneous lytic cell death (Fig. 3A and B, Supporting Information Fig. 9C). However, in line with the observations from primary cells from STAT3-deficient patients, ST18-treated cells showed significantly augmented LDH release upon challenge with *S. aureus*. Similar to the observation obtained from patient samples, the supplementary lytic effect imposed by

Table 1. Laboratory findings and demographic details of the study cohort

Patient-ID	HIES #1	HIES #2	HIES #3	HIES #4	HIES #5	HIES #6	HIES #7	HIES #8
Age	11 years	12 years	15 years	16 years	4 years	26 years	30 years	2 years
Gender	Male	Female	Male	Male	Male	Female	Male	Male
Mutation	H332L	R382L	T622I	K340E	R382W	Y657C	R382L	D371_G380del
Localisation of Mutation	DNA-binding domain	DNA-binding domain	SH2 domain	DNA-binding domain	DNA-binding domain	SH2-binding domain	DNA-binding domain	DNA-binding domain
Age at first symptoms	Newborn period	Newborn period	Newborn period	Newborn period	8 weeks	Newborn period	6 months	3 months
First clinical symptom of HIES	Newborn eczema	Newborn eczema	Newborn eczema	Newborn eczema/ impetigo	Superinfected eczema	Infected eczema	Skin abscesses	Skin abscesses
Age when HIES was first suspected	7 years	1 year	6 years	5 years	3 years	19 years	2 years	6 months
Which symptom led to investigation for HIES?	Recurrent abscesses and herpes zoster	Recurrent purulent otitis, eczema, cold abscesses	Pleuropneumonia and pneumatocele, elevated IgE	Recurrent pulmonary infections, skin eczema	Recurrent skin infections	Recurrent abscesses, skin infections	Abscesses	Skin abscess
Medical care in immunological clinic?	Since age 7 years	Since age 7 years	Since age 6 years	Since age 15 years	Since age 3 years	Since age 20 years	Since age 18 years	Since age 6 months
Documented neutrophil numbers	Some normal but also several low values (range 577–1270/μL)	Normal	Normal	Normal	Generally normal, some low values (range 740–990/μL)	Normal	Normal	Normal
Eosinophil numbers (highest documented value)	352/μL	800/μL	800/μL	790/μL	3180/μL	2500/μL	1400/μL	2200/μL
Exemplary % eosinophils among granulocytes documented during experiments	19%	23%	10%	19%	27%	17%	20%	40%
IgE (highest documented value)	1040 kU/L	4101 kU/L	17 081 kU/L	9965 kU/L	1997 kU/L	12 640 kU/L	36 000 kU/L	4270 kU/L
IgG, IgA, IgM	Normal	Normal	Not determined	Normal	Normal	Normal	Normal	Normal [41]
Protein specific antibodies	Anti-tetanus after booster: normal	Anti-tetanus after booster: absent; anti-MMR after booster: normal	Anti-tetanus, anti-measles and anti-varicella: detectable	Anti-diphtheria and anti-tetanus after booster: absent; anti-mumps: absent	Anti-tetanus after booster: absent	Anti-diphtheria: normal; Anti-tetanus: low	Anti-tetanus: normal	Anti-tetanus: low; anti-haemophilus: low
Anti-pneumococcal antibodies	Partial positive vaccination titres	Partial positive vaccination titres	Partial positive vaccination titres	Low	Not performed	Low	Normal	Normal
T$_{H}$17 cells/IL-17 formation	Almost absent (0.04% CD3+CD4+IL-17+ cells)	Almost absent (0.01% CD4+CD45RO+IL-17+ cells)	Almost absent (0.07% CD3+CD4+IL-17+ cells)	Low (0.2% CD3+CD4+IL-17+ cells)	Low (0.27% CD4+CD45RO+ IL-17+ cells)	Low IL-17 release from PBMCs [35]	Low IL-17 release from PBMCs [35]	LOW (0.5-1% CXCR-CCR4+CCR6+) [41]
B cell differentiation	Slightly low percentage of marginal zone B cells (4.28%), reduced percentage of class-switched memory B cells (2.92%)	Low percentage of marginal zone B cells (3.78%), normal percentage of class-switched memory B cells (5.81%).	Low marginal zone B cells (3.61%), almost complete lack of CD27+IgD+ and class-switched CD27+IgD− memory B cells	Low percentage of marginal zone B cells (3.95%) and very low class-switched memory B cells (0.96%)	Low numbers of marginal zone B cells (4.03%) and of class-switched CD27+IgD− B cells (2.13%)	Not performed	Not performed	Low levels of marginal zone B cells (2%) and switched memory B cells (0.5-1%) [41]
FOX P3 expression	Normal	Normal	Normal	Normal	Not performed	Not performed	Not performed	Not performed

DNA, deoxyribonucleic acid; FOX P3, forkhead box P3; HIES, autosomal-dominant hyper-IgE syndrome; IL-17, Interleukin-17; MMR, measles, mumps, rubella; SH2, Src Homology 2; [] = literature reference.
Reference values:
- Neutrophil numbers were considered normal if they were > 1500/μL.
- Eosinophilia (i.e. elevated blood eosinophil counts) was considered if eosinophil numbers were > 500/μL.
- Markers to detect TH17 cells varied. Internal reference values from full-aged, healthy individuals without STAT3 mutations as provided by the performing laboratory are as follows: CD3+CD4+IL-17+ cells: 0.38-1.1%. CD4+CD45RO+IL-17+ cells: 1.1-4.76%. CXCR-CCR4+CCR6+ cells: 3.8-5.6%.

Table 2. Clinical presentation and complications

Patient-ID	HIES #1	HIES #2	HIES #3	HIES #4	HIES #5	HIES #6	HIES #7	HIES #8
Pneumonia	Recurrent obstructive bronchitis/pneumonia below the age of 2 years	ARDS due to RSV-pneumonia requiring ventilation at age of 5 months. Pneumonia at age 1, 2, 9, and 14 years	8 pneumonia (at age 4, 6, 7, 9, 10, and 15 years), 2 times with isolation of H. influenzae	6–7 pneumonia/year between age 0-5 years, 1 pneumonia/year since age 5 years, overall over 30 episodes	None	None	1 pneumonia at age 30 years	None
Skin abscesses	recurrent abscesses various locations: armpit, groin, antecubital fossa, buttocks	twice (forehead, forearm)	twice (buttocks, axilla)	six times (knee, eyelids)	superinfected eczema, recurrent panaritia	recurrent, location not documented	once, location not documented	recurrent, first time at age 3 months (S. aureus)
Abscesses of inner organs	Lymph node abscess	None	Lung abscess requiring drainage and subsequent resection of upper lung lobe	Not documented	Lymph node abscess, due to Streptococcus pyogenes	Not documented	Not documented	No
Other abscesses and skin infections	Recurrent panaritia	Abscess of labium	Dental abscess requiring tooth extraction	Not documented	Not documented	Hip joint	Not documented	No
Otitis media	Recurrent, up to > 10/year. Chronic purulent otitis at age 2 years. Mastoiditis due to S. pneumonia and S. aureus at age 3 years.	Recurrent otitis. Documented colonization with Pseudomonas aeruginosa	Once	Once	5–6 episodes/year at age 2 and 3 years	Yes, recurrent	Not documented	No
Specific viral infections	Severe varicella at age 6 months, once herpes zoster	Varicella infection	H1N1-Infection with central pneumonia and P. aeruginosa colonization	No	No	No	No	No
Positive S. aureus findings	Isolated from otorrhea	Isolated from abscesses, recurrent colonization with MRSA	Isolated from abscesses, recurrent colonization in bronchoalveolar lavage, skin colonization	Skin colonization	Skin colonization	Documented from skin, nose, throat	Not documented	Isolated from abscesses
Positive S. pneumoniae findings	Not documented	Not documented	Sputum	Once in bronchoalveolar lavage	Lymph node abscess	Yes, recurrent	Not documented	Not documented
Positive fungal findings	Oral candidiasis, nail mycosis	Genital candidiasis, nail mycosis, oral candidiasis	Oral candidiasis	Tinea pedis, nail mycosis	Oral candidiasis	Nails	Aspergillus, Candida and Scedosporium in the lung	Oral candidiasis; Aspergillus in the nasopharynx
Positive Haemophilus influenzae findings	Not documented	Sputum	Twice during pneumonia, once during bronchitis (sputum)	Not documented	Once during otitis	Yes, recurrent	Yes	Once in nasopharynx during acute respiratory infection
Positive Pseudomonas aeruginosa findings	Not documented	Recurrently documented during otitis	Only colonization	Colonization	Not documented	No	Chronic colonization of the lungs	No
Pneumatocele	No	Yes	Yes (left and right lung)	Yes (right lung)	No	No	No	No
Bronchiectasis	No	No	Yes	Yes	No	No	Yes, progressive	No
Clubbed fingers/ hippocratic nails	No	No	No	Yes	No	No	No	No

(Continued)

Table 2. Continued

Patient-ID	HIES #1	HIES #2	HIES #3	HIES #4	HIES #5	HIES #6	HIES #7	HIES #8
Bronchoalveolar lavage (BAL)-results (as documented by the diagnostic pathology department)	Not performed	No growth	During pneumonia: numerous neutrophils, lymphocytes and some macrophages, sparse S. aureus, very few eosinophils. Numerous degenerative transformed macrophages and neutrophils.	Prior to lung resection: numerous granulocytes, no bacteria. After lung resection: S.pneumoniae, numerous granulocytes, numerous squamous epithelia.	Not performed	Not performed	Not performed	Not performed
Histology (as documented by the diagnostic pathology department)	Lymph node abscess: granulocytic absceding inflammation with histiocytic reaction	Skin biopsy: inflammatory infiltration of lymphatic and histiocytic cells	Dental abscess: phlegmonous inflammation with presence of numerous neutrophils, some lymphocytes and plasma cells, few eosinophils (Supporting Information Fig. 1A). Lung abscess: purulent, absceding pneumonia with massive numbers of alveolar macrophages, histiocytes, neutrophils, cell detritus. On the edges, fibrinoid connective tissue with overall fewer cells, but several eosinophils, plasma cells and neutrophils. In bronchia: cell detritus, foam cell macrophages and neutrophils (Supporting Information Fig. 1B).	Lung resection (not during acute infection): findings are compatible with multiple past pneumonia. No significant eosinophilia, no granuloma, no acute purulent infection, no fungal infection.	Lymph node aspiration: immature suppurative granuloma reaction. Multiple eosinophils in the granuloma center (Supporting Information Fig. 1C and D).	Not performed	Not performed	Pyogenic granuloma on the tongue

ARDS, acute respiratory distress syndrome; RSV, respiratory syncytial virus.
The data in this table was retrieved from patient charts and/or questionnaire.

Table 3. Assessed neutrophil functions

Patient-ID	HIES #1	HIES #2	HIES #3	HIES #4	HIES #5	HIES #6	HIES #7	HIES #8
Neutrophil chemotaxis	Normal	n.p.	Normal	n.p.	n.p.	n.p.	n.p.	n.p.
Neutrophil adhesion	Normal	n.p.	Normal	n.p.	n.p.	n.p.	n.p.	n.p.
Quantitative NET formation	Normal	Normal	Normal	Normal	Normal	n.p.	n.p.	n.p.
ROS release upon PMA stimulation	Normal	Normal	Normal	n.p.	Normal	n.p.	n.p.	Normal [41]
Whole blood-killing of S. aureus	Normal	Normal	n.p.	n.p.	Normal	Normal	Normal	Normal
Neutrophil-killing of S. aureus	n.p.	Normal	n.p.	n.p.	n.p.	Normal	Normal	Normal

n.p., test not performed; [], literature reference.

eosinophils during staphylococcal infection was also present in healthy controls (Fig. 3A). When evaluating whether presence of eosinophils during granulocyte isolation in healthy controls affected the LDH release, a mild correlation was observed for the percentage of eosinophils within the granulocyte fraction and the LDH release of DMSO-treated granulocytes stimulated with vehicle or *S. aureus* (Supporting Information Fig. 9A and B). The correlation was lost after preincubation with ST18, which led to a significant increase in LDH upon *S. aureus* challenge (Supporting Information Fig. 9C).

The staphylococcal agr-system influences cell lysis during STAT3 inhibition

Secreted virulence factors such as hemolysins and leukocidins have been implicated in mediating the lytic effect of *S. aureus* on eosinophils and neutrophils [21, 22]. Therefore, we studied whether the expression of secreted virulence factors was essential for the observed cell lysis in STAT3-inhibited samples. Granulocytes from healthy blood donors were stimulated with either an *agr*+ wild type *S. aureus* strain (8325-4 [WT]) or its isogenic *agr*− mutant (WA250 [*agr*−]) in the presence or absence of STAT3 inhibitor. The *agr*− strain is characterized by reduced expression of α-, β-, δ-, and γ-hemolysins, leukocidins, enterotoxins, and TSST-1 [23]. Challenge with the *agr*− strain diminished LDH release in both STAT3-inhibited and control samples (Fig. 3B). The highest cell lysis was observed in STAT3-inhibited samples treated with wild type bacteria expressing the *agr* gene regulator.

STAT3 inhibition in granulocytes does not affect the release of IL-1β, TNF-α, or IL-8 by *S. aureus*

Different cytokines implicated in cell death were analyzed in supernatants of STAT3-inhibited and control granulocytes following incubation with WT and *agr*− *S. aureus*. IL-1β or TNF-α were not detected in any of the samples (three independent experiments with six different controls, detection limit IL-1β 3.9 pg/mL, TNF-α 15.6 pg/mL). However, IL-8 production was more pronounced when stimulated with the *agr*− in comparison to the WT strain (Fig. 3C). Still, IL-8 production did not differ significantly between STAT3-inhibited and control samples.

Discussion

STAT3-deficient HIES patient are strikingly susceptible to acquire staphylococcal infections and suffer from enhanced tissue destruction following pulmonary infections. So far, this specific susceptibility remains incompletely understood. Given the role of neutrophils in the defense against *S. aureus* and *Candida*, a specific dysfunction in STAT3-deficient neutrophils would offer a logical solution [11–13]. However, in agreement with other recent studies we found normal superoxide production [1, 3, 24],
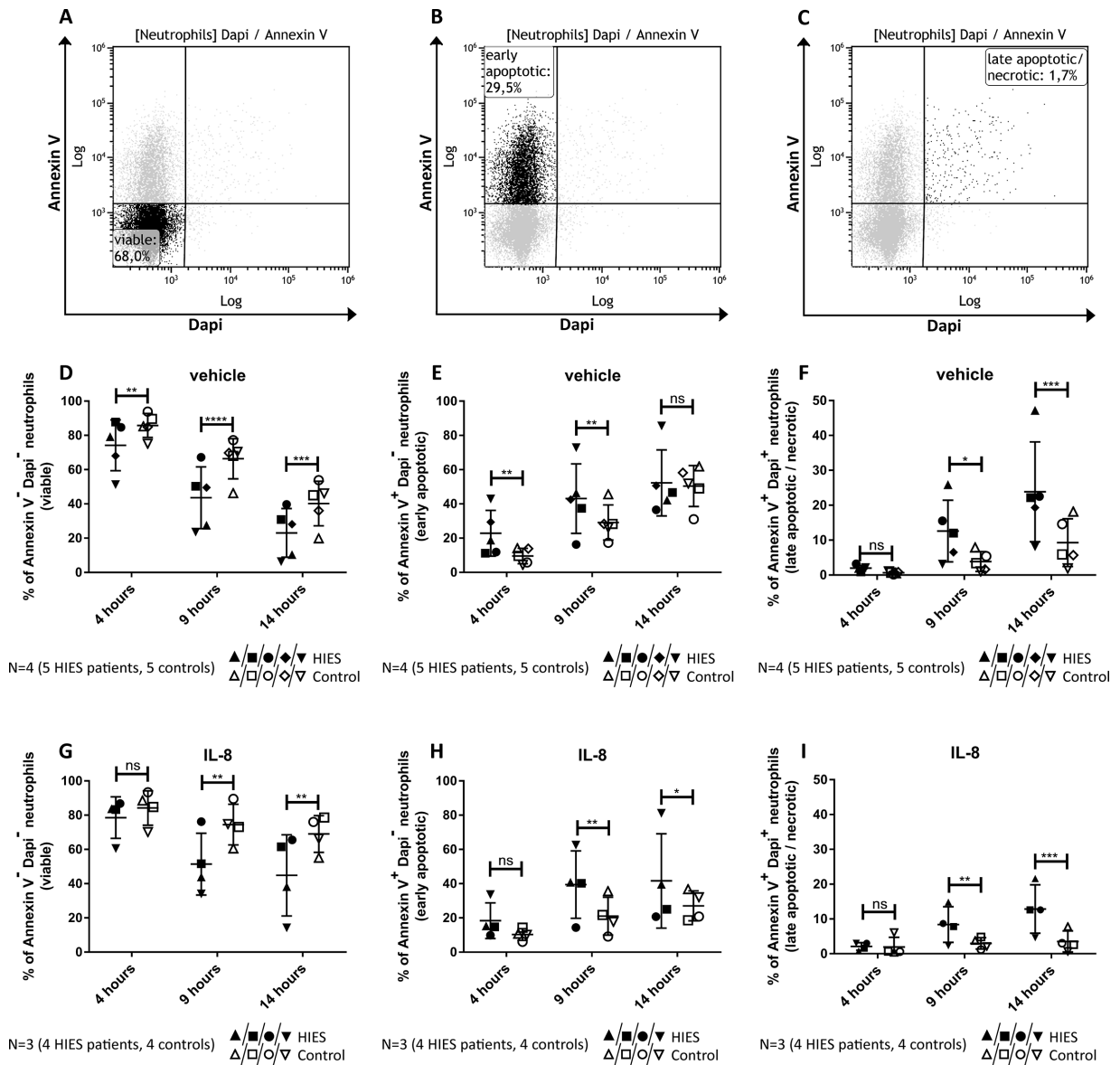
Figure 1. Accelerated cell death kinetics of neutrophils from STAT3-deficient HIES patients. Granulocytes were isolated from STAT3-deficient HIES patients and concomitant control samples followed by cytokine or vehicle stimulation. At designated time points (four, nine, and fourteen hours), equal amounts of the samples were stained with Annexin V and Dapi and analyzed by flow cytometry (detailed gating strategy see Supporting Fig. 11). (A–C) displays the same dataset highlighting the gating strategy for the identification of the following cell death categories according to the respective Annexin V and Dapi staining: (A) viable (Annexin V⁻ Dapi⁻), (B) early apoptotic (Annexin V⁺ Dapi⁻) and (C) late apoptotic/necrotic (Annexin V⁺ Dapi⁺) neutrophils. Primary necrotic (Annexin V⁻ Dapi⁺) neutrophils are not displayed since no significant differences were found. (D–I) Quantitative data showing the percentages of (D, G) viable, (E, H) early apoptotic and (F, I) late apoptotic/necrotic neutrophils after stimulation with (D–F) vehicle or (G–I) Il-8. (D–I) Data are shown as mean ± SD and are pooled from four (D–F) and three (G–I) independent experiments, respectively. Each patient was matched to the concomitant daily control. Matched samples are displayed with the same symbol (dark symbols = HIES patients; light symbols = controls). RM (repeated measures) two-way Anova with Bonferroni-Analysis for multiple comparison was used for statistical analysis ($p > 0.05$ = not significant (ns); $p \leq 0.05$ = *; $p < 0.01$ = **; $p < 0.001$ = ***; $p < 0.0001$ = ****).

chemotaxis [3], and preserved staphylococcal killing [1]. Moreover, tissue samples and BAL fluid from our patients revealed presence of neutrophils during infections, thus underscoring functional chemotaxis, despite low or absent IL-17 signaling. This observation and the maintained infectious phenotype of STAT3-deficient individuals with somatic mosaicism and a normal $T_H 17$ compartment [9] suggest that additional factors are crucial for their susceptibility to infection.

The majority of HIES patients present with eosinophilia, which was also true for seven of our eight patients. However, even in the absence of blood eosinophilia, the percentage of eosinophils among isolated granulocytes was generally elevated in patients.
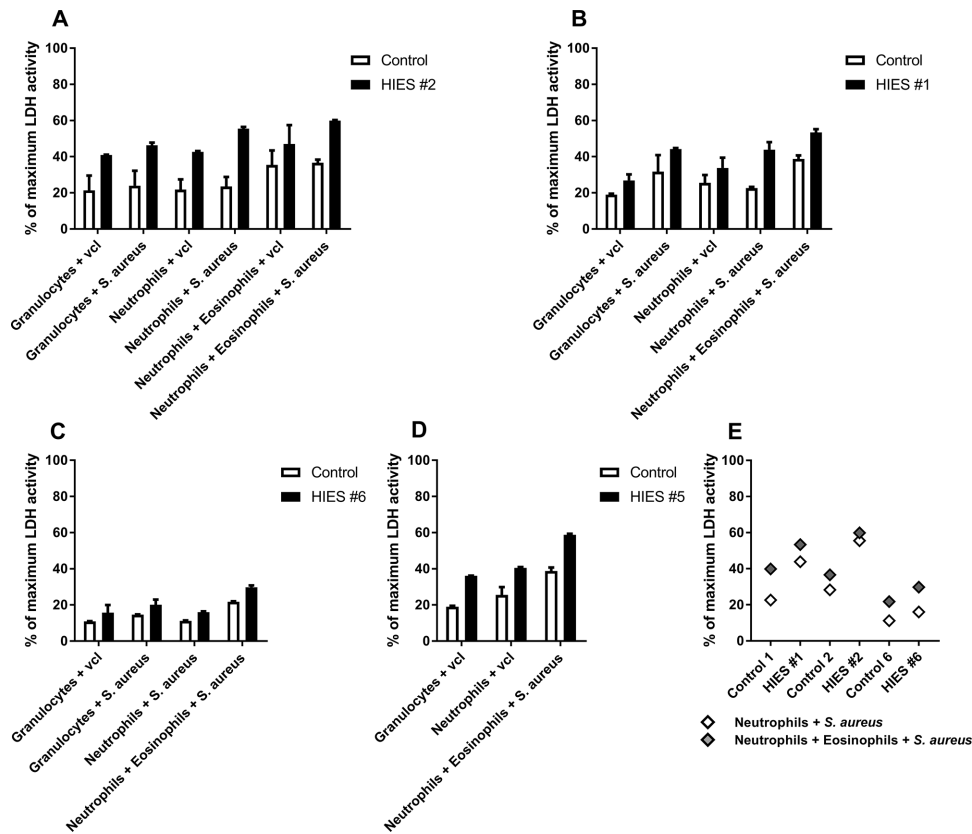
Figure 2. Augmented spontaneous and *S. aureus*-induced lytic cell death in STAT3-deficient HIES patients. Eosinophils and neutrophils were purified by magnetic cell separation from granulocyte suspensions of STAT3-deficient HIES patients and concomitant healthy controls. LDH release was measured in supernatants five hours after costimulation of various granulocyte fractions (original granulocyte suspension, highly purified neutrophils, purified neutrophils + 20% purified eosinophils) with vcl (vehicle = medium) or *S. aureus* WT (MOI 5). Due to limitations in cell availability not all conditions could be performed in every experiment. (A–D) Three individual experiments are shown, including cells from four different HIES patients and their concomitant controls. Depicted is the mean with SD of at least two sample replicates. B+D were performed at the same time point and contain the same controls. (E) The effect of supplementation of neutrophils with eosinophils on *S. aureus*-induced cell lysis for available samples in A–C is shown. Data are pooled from three independent experiments.

Analyzed neutrophil cell death kinetics in vitro were accelerated in HIES patients in the presence of high eosinophil numbers in the granulocyte preparations. This effect was attributable to both enhanced early apoptosis and late apoptosis/necrosis. As the percentage of eosinophils correlated only at initial time points with the percentage of early apoptotic and late apoptotic/necrotic neutrophils, eosinophilia may most likely only partly explain the accelerated cell death kinetics seen in vitro. Because apoptotic neutrophils are functionally impaired in vitro [25], accelerated cell death may become relevant in vivo, particularly during infections when neutrophil survival is prolonged by cytokines. Consistent with our findings, it was previously reported that STAT3-deficient neutrophils showed enhanced proinflammatory cytokine expression, yet, without the expected associated apoptosis delay [1]. In our hands, patient neutrophils responded with delayed apoptosis to IL-8, GM-CSF, and G-CSF. Yet, cell death was still clearly enhanced when compared to control neutrophils, particularly at late time points. The partially maintained anti-apoptotic proper-

ties are probably related to residual STAT3 signaling in affected individuals, as well as alternative signaling pathways [26–28].

Given the relevance of *S. aureus* infections in HIES patients, we assessed how stimulation with *S. aureus* would affect neutrophil fate in the presence of STAT3 deficiency and eosinophils. In the limited number of available samples we observed that granulocytes and purified neutrophils from STAT3-deficient HIES patients showed an increased baseline of spontaneous cell lysis and were more sensitive to *S. aureus*-induced lytic cell death than the respective control cells. Supplementation of purified neutrophils with eosinophils led to higher *S. aureus*-induced lytic cell death in both HIES patients and controls. This suggests that the observed accelerated cell death rate in the context of high levels of eosinophils in vitro may also play a role in staphylococcal infections. In our cohort, eosinophils were documented in three histological samples and one BAL obtained during acute infections. Others have reported on eosinophilic infiltrations in the lung, skin or abscesses in individuals with STAT3-deficient HIES [24, 29]. Interestingly,
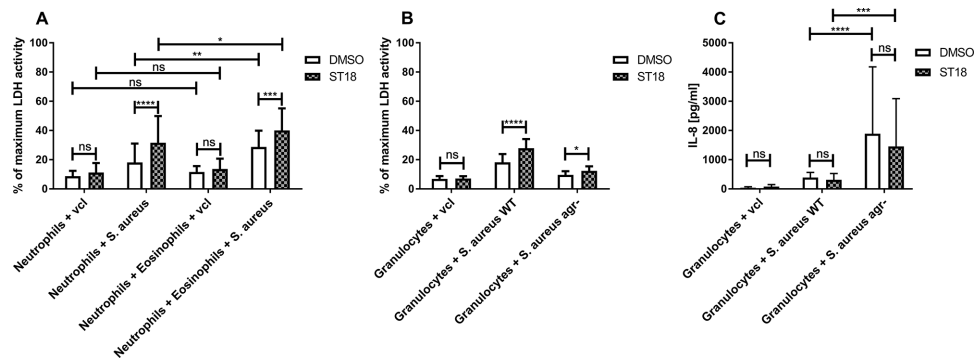
Figure 3. Increased *S. aureus*-induced lytic cell death in STAT3-inhibited cells. Eosinophils and neutrophils were purified by magnetic cell separation from healthy control granulocyte suspensions. The original granulocyte suspension and/or purified neutrophils were incubated for 60 min with 20 μM ST18 (STAT3 Inhibitor XVIII, BP-1-102) or DMSO. Subsequently, cells were washed and coincubated with vcl (vehicle = medium), *S. aureus* WT and/or *S. aureus* agr⁻ (both MOI 5) for five hours. (A) LDH release after stimulation of purified STAT3-inhibited (ST18) and control (DMSO) neutrophils with/without supplementation of 20% eosinophils with vcl or *S. aureus* WT for five hours is shown. Displayed are pooled results from eight independent experiments with cells from nine different controls. (B) LDH release following stimulation of STAT3-inhibited (ST18) and control (DMSO) granulocytes with vcl, *S. aureus* WT, or *S. aureus* agr⁻ (both MOI 5) for five hours is shown. Displayed are pooled results from four independent experiments with cells from eight different controls. (C) IL-8 release of STAT3-inhibited (ST18) and control (DMSO) granulocytes following stimulation with vcl, *S. aureus* WT, or agr- (both MOI 5) for five hours is shown. Displayed are pooled results from three independent experiments with six different controls (detection limit 3.1 pg/mL). Statistical analysis A–C: Depicted is the mean with SD. RM (repeated measures) two-way Anova with Bonferroni-Analysis for multiple comparison was used for statistical analysis for A–C ($p > 0.05$ = not significant (ns); $p \leq 0.05$ = *; $p < 0.01$ = **; $p < 0.001$ = ***; $p < 0.0001$ = ****).

eosinophils appear to be particularly susceptible to cell lysis and degranulation when incubated with *S. aureus* [20, 21]. In our experiments employing eosinophil supplementation to control neutrophils, increased cell lysis became evident when stimulating with *S. aureus*. Therefore, local eosinophilia could potentially limit antimicrobial efficacy of neutrophils during manifest infection.

The use of a chemical STAT3 inhibitor allowed assessing the functional consequences of STAT3 deficiency in the absence of high numbers of eosinophils during sample preparation. While STAT3 inhibition alone did not increase cell lysis, there was a significant augmentation of *S. aureus*-induced lytic cell death in STAT3-inhibited cells. The initial concentration of eosinophils did only mildly correlate with cell lysis of granulocytes and the correlation was completely lost after STAT3-inhibition. This suggests that reduced STAT3 signaling in neutrophils may negatively affect cell survival during staphylococcal infection, independent of eosinophilia.

It has been argued that increased neutrophil lysis by certain *S. aureus* strains favors pathogen survival and augments disease severity in otherwise healthy individuals [30]. The staphylococcal quorum sensing agr-operon activates transcription and production of virulence factors that may destroy neutrophils [22]. We demonstrate that the effect of reduced STAT3 signaling on cell death is most pronounced in the presence of secreted staphylococcal virulence factors present in the *agr*⁺ wild type strain. Enhanced anti-apoptotic IL-8 induction may partly explain the protection from cell death during incubation with the toxin-deficient *agr*⁻ mutant. Yet, this does not appear to be relevant for the observed differences in cell death during deficient STAT3 signalling, as IL-8 expression did not differ between STAT3-inhibited and control samples. The precise mechanism underlying the increased *S. aureus*-induced cell

lysis in STAT3 deficiency remains to be elucidated. Nevertheless, our findings may be clinically relevant, as a recent study showed that HIES patients are frequently colonized with highly virulent and antibiotic-resistant *S. aureus* strains [31].

STAT3 is ubiquitously expressed. Thus, the specific infection susceptibility in HIES at mucocutaneous sites, is likely shaped by both immune and epithelial cells. Potential epithelial factors include altered cytokine expression and deficient release of antimicrobial factors [32–34]. Together with impaired IL-17 formation [7, 35] these factors could lead to a higher bacterial load at the infection site. In this setting, STAT3-deficient neutrophils that respond with increased cell lysis to virulent *S. aureus* may further impair infection control and contribute to tissue destruction. Maintained neutrophil functionality in HIES, however, seems to be sufficient to prevent invasive organ and blood stream infections.

Our results also offer an explanation for the controversial literature on neutrophil functions (e.g. chemotaxis) in HIES. Density gradient centrifugation with subsequent erythrocyte lysis is the most widely used method for rapid isolation of neutrophils [30, 36, 37]. Red blood cell lysis with ammonium chloride is beneficial for maintenance of neutrophil properties [37], but may impair eosinophil viability and induce eosinophil degranulation [38, 39]. While this effect may be negligible in donors with low eosinophil contamination, a high eosinophil percentage in granulocyte preparations, as in HIES, negatively affects neutrophil viability and may then alter neutrophil function analysis, particularly if analysis is delayed [40].

Limitations of our study include the restricted number of patients and that not all experiments could be performed for each patient due to limited clinical material. Yet, despite interindividual variability our results were consistent. Moreover, the

effect of eosinophil supplementation and STAT3 inhibition was reproducible in healthy controls. Still, the small sample size impedes correlating experimental findings with disease severity in individual patients. Furthermore, although neutrophils and eosinophils were detected in histological samples from our patients, their interaction in vivo may be affected by additional factors, which are not present during in vitro testing. It would be particular interesting to further evaluate how the specific milieu induced in STAT3-deficient lung epithelium may alter the host-pathogen interactions and affect postinfectious tissue remodeling.

In summary, we describe eight unrelated STAT3-deficient HIES patients with variable clinical presentation and overall normal basic neutrophil functions in vitro, in spite of staphylococcal infections and mucocutaneous candidiasis. Histological samples in individual patients indicate that neutrophils are recruited to infected tissues, even though IL-17 formation is impaired. Our data suggest that both STAT3 deficiency and the presence of eosinophils negatively affect neutrophil cell death kinetics, which may hamper in vitro neutrophil function testing in STAT3-deficient patients. The presence of eosinophils in abscesses from HIES-patients and the observed increased *S. aureus*-induced cell lysis in presence of eosinophils and STAT3 deficiency offer the possibility that this effect may also be relevant during infections. Further investigations are needed to decipher the precise molecular mechanisms behind this observation and to assess whether increased *S. aureus*-induced cell death in the presence of STAT3 deficiency or eosinophilia affects pathogen control and tissue destruction in vivo.

Materials and methods

Study cohort and ethics

The study included eight patients with genetically confirmed STAT3-deficient HIES at the Centre of Chronic Immunodeficiency, Freiburg, Germany or at the Karolinska University Hospital Stockholm, Sweden. Ethical approval was obtained by local ethical committees. All patients or their legal guardians gave informed consent for participation and data collection. Clinical data were retrieved from questionnaires filled in by patients or their parents, and by reviewing patient charts. Patients #6 and #7 participated earlier in another study [35]. Details on patient #8 were reported previously [41]. Blood samples from healthy controls were obtained either anonymously from the blood bank, or following informed consent from unaffected relatives, laboratory or hospital staff. For experiments requiring eosinophils, blood samples were also obtained from healthy donors without infectious history, but with supposedly asthmatic or allergic predisposition not requiring current medication, following informed consent. Here, sample blood volume was increased to 80 mL to achieve sufficient eosinophil numbers for experiments.

Granulocyte isolation and purification of neutrophils and eosinophils

Blood samples were processed within three hours after blood draw using a standard protocol for neutrophil isolation [36, 37]. Each patient sample was accompanied by a concomitant control sample. Samples were processed via density gradient centrifugation with isotonic Biocoll with 10 mm hydroxyethyl-piperazineethane-sulfonic acid buffer (HEPES) (density of 1.077 g/mL, Biochrom). Red cells were lysed with lysis buffer containing ammonium chloride, potassium hydrogen carbonate, and ethylenediaminetetraacetic acid (EDTA) (PH 7.4) at 4°C, followed by washes in PBS and final suspension in medium, i.e. sterile-filtered RPMI-1640 (without Phenolred, endotoxin tested; Sigma-Aldrich) + 2% human albumin (Albunorm 20%, Octapharma) unless otherwise indicated. Viability and purity of the obtained granulocytes was routinely ≥ 95% as assessed by trypan blue staining and flow cytometry, respectively. In healthy controls, contamination of granulocytes with eosinophils was usually < 7%. For selected experiments, granulocytes were further purified into neutrophils and eosinophils employing the EasySep Human Neutrophil Enrichment kit (Stemcell) and the EasySep Human Eosinophil Enrichment kit (Stemcell), generally achieving a purity ≥ 99% for neutrophils and ≥ 94% for eosinophils (Verification of cell origin see Supporting Information Fig. 10A–F). In order to highlight the differences between the preparations, we use the term *granulocytes* when referring to the initial isolation product and *neutrophils* when further purification steps were employed.

Bacterial strains

We used *S. aureus* strain 8325-4 (WT) and its isogenic *agr*-mutant WA250 (*agr⁻*) with known lower toxin and enzyme expression [23].

Granulocyte cell death kinetics by flow cytometry

Granulocytes were stimulated with medium (vehicle = vcl) or cytokines (IL-8, G-CSF, GM-CSF; PeproTech or R&D Systems). At designated time points, equal cell numbers were stained with Annexin V (BD Bioscience) and 4′, 6-diamidino-2-phenylindole dihydrochloride (Dapi, Life Technologies) to detect apoptotic and necrotic cells, respectively. The following categories were employed: early apoptotic (Annexin V⁺ Dapi⁻) and late apoptotic/necrotic (Annexin V⁺ Dapi⁺), primary necrotic (Annexin V⁻ Dapi⁺) and viable (Annexin V⁻ Dapi⁻) neutrophils. For gating strategy see Supporting Information Fig. 11. Neutrophils were generally distinguished from eosinophils by means of their different autofluorescence, e.g. in FL3 (excitation 488 nm; emission filter 620/30) versus FL4 (excitation 488 nm; emission filter 695/30). Samples were analyzed on a Gallios flow cytometer (Beckman Coulter). No additional antibodies were used during cell death assessment. Cell type origin in the autofluorescent gates

was confirmed in separate experiments (Supporting Information Fig. 10D–F). Flowcytometric data was analyzed with Kaluza software (Beckman Coulter).

Lytic cell death determination by LDH release

In order to assess spontaneous and *S. aureus*-induced lytic cell death, various granulocyte fractions (original granulocyte suspension, purified neutrophils and/or purified neutrophils + 20% eosinophils) were incubated with vehicle (medium), *S. aureus* WT and/or *agr*− mutant at a multiplicity of infection (MOI) of five for five hours at 37°C, 5% CO2. After two hours, gentamicin (final concentration 200 μg/mL) was added to kill off extracellular bacteria. After centrifugation the obtained supernatant was used for analysis of LDH and cytokines. LDH release was measured with the Cytotoxicity Detection Kit^PLUS (Roche) according to the manufacturer's instructions.

STAT3 inhibition of neutrophils from healthy controls

Granulocytes or purified neutrophils were incubated for one hour with the STAT3 Inhibitor XVIII, BP-1-102 (Calbiochem) here named ST18, at a concentration of 20 μM (Supporting Information Figs. 12 and 13) or DMSO diluted in medium as vehicle control, followed by two washes with PBS. Subsequently, spontaneous and *S. aureus*-induced lytic cell death of various granulocyte fractions was determined as described above.

Measurement of cytokines

IL-8, IL-1beta, and TNF-alpha were measured in supernatants obtained after five hour incubation of STAT3-inhibited or DMSO-treated control granulocytes with medium (vcl), *S. aureus* WT or *agr*− employing commercial ELISA-kits (BD Bioscience OptEIA ELISA kits).

NET quantification and visualization

Granulocytes were seeded in black 96-well plates and stimulated with vehicle or 25 nM phorbol 12-myristate 13-acetate (PMA, Sigma Aldrich) at 37°C, 5% CO2 for 2.5 hours. Then, the cell impermeable DNA staining dye Sytox green (Invitrogen) was added and fluorescence signal was detected with a fluorescent reader. NET formation was visually confirmed by fluorescence microscopy (Supporting Information Fig. 2). In selected experiments, granulocytes were stained with CellTracker^TM dye (Invitrogen) prior to stimulation. Following two hours of stimulation, Sytox green was added and the plate was analyzed with an automated fluorescence microscope (Olympus Scan^R) (Supporting Information Figs. 3 and 4).

Staphylococcal killing assay

Fifty microliters whole blood and/ or 2×10^5 granulocytes in RPMI-1640 + 2% human albumin were incubated with serum-opsonized *S. aureus* (2×10^5 cfu, MOI 1) at 37°C, 5% CO_2, 130 rpm. After 30 and 120 minutes cells were lysed with 0.1% Saponin and bacterial counts were determined via serial dilution plating.

Neutrophil function tests: ROS, chemotaxis, and adhesion

Basic neutrophil function tests were performed as previously described [42]. In brief, PMA-induced release of ROS was determined by lucigenin chemiluminescence assay. For the adhesion assay, Calcein-AM (Life Technologies) stained cells were stimulated with vehicle, Pam3Cys (Invitrogen), n-formyl-L-methionyl-leucine-phenylalanine (fMLP), PMA, or LPS (all Sigma Aldrich) for 30 minutes, then washed and lysed, followed by detection of fluorescence intensity. For chemotaxis, Calcein-AM stained cells were seeded in Fluoroblok-inserts (BD Falcon). Chemotactic substances such as fMLP, platelet activating factor (PAF), and C5a (all Sigma Aldrich) were pipetted onto the plate bottoms and chemotaxis of cells from the upper insert to the bottom was determined by measuring fluorescence intensity over 45 minutes. Due to limitations in the availability and size of patient samples, not all tests could be performed for every patient.

Statistics

Statistical analysis was performed using GraphPad Prism 7. Patient and concomitant control samples and in vitro treated control samples were matched and analyzed with repeated measures (RM) two-way Anova with Bonferroni-Analysis for multiple comparison. Statisical analysis was only performed if ≥ three sample sets were available. Correlation was estimated using Pearson correlation.

(BMBF 01EO 0803) and the German Society of Pediatric Infectious Disease (DGPI). None of the funding sources was involved in study design, collection, analysis, and interpretation of data or writing of the report.

Conflict of interest: None.

References

1 Holland, S. M., DeLeo, F. R., Elloumi, H. Z., Hsu, A. P., Uzel, G., Brodsky, N., Freeman, A. F. et al., STAT3 mutations in the hyper-IgE syndrome. *N. Engl. J. Med.* 2007. **357**: 1608–1619.

2 Minegishi, Y., Saito, M., Tsuchiya, S., Tsuge, I., Takada, H., Hara, T., Kawamura, N. et al., Dominant-negative mutations in the DNA-binding domain of STAT3 cause hyper-IgE syndrome. *Nature* 2007. **448**: 1058–1062.

3 Chandesris, M. O., Melki, I., Natividad, A., Puel, A., Fieschi, C., Yun, L., Thumerelle, C. et al., Autosomal dominant STAT3 deficiency and hyper-IgE syndrome: molecular, cellular, and clinical features from a French national survey. *Medicine* 2012. **91**: e1–19.

4 Freeman, A. F., Kleiner, D. E., Nadiminti, H., Davis, J., Quezado, M., Anderson, V., Puck, J. M. et al., Causes of death in hyper-IgE syndrome. *J. Allergy Clin. Immunol.* 2007. **119**: 1234–1240.

5 Ma, C. S., Chew, G. Y., Simpson, N., Priyadarshi, A., Wong, M., Grimbacher, B., Fulcher, D. A. et al., Deficiency of Th17 cells in hyper IgE syndrome due to mutations in STAT3. *J. Exp. Med.* 2008. **205**: 1551–1557.

6 Milner, J. D., Brenchley, J. M., Laurence, A., Freeman, A. F., Hill, B. J., Elias, K. M., Kanno, Y. et al., Impaired T(H)17 cell differentiation in subjects with autosomal dominant hyper-IgE syndrome. *Nature* 2008. **452**: 773–776.

7 Minegishi, Y., Saito, M., Nagasawa, M., Takada, H., Hara, T., Tsuchiya, S., Agematsu, K. et al., Molecular explanation for the contradiction between systemic Th17 defect and localized bacterial infection in hyper-IgE syndrome. *J. Exp. Med.* 2009. **206**: 1291–1301.

8 Puel, A., Cypowyj, S., Marodi, L., Abel, L., Picard, C. and Casanova, J. L., Inborn errors of human IL-17 immunity underlie chronic mucocutaneous candidiasis. *Curr. Opin. Allergy Clin. Immunol.* 2012. **12**: 616–622.

9 Hsu, A. P., Sowerwine, K. J., Lawrence, M. G., Davis, J., Henderson, C. J., Zarember, K. A., Garofalo, M. et al., Intermediate phenotypes in patients with autosomal dominant hyper-IgE syndrome caused by somatic mosaicism. *J. Allergy Clin. Immunol.* 2013. **131**: 1586–1593.

10 Davis, S. D., Schaller, J. and Wedgwood, R. J., Job's Syndrome. Recurrent, "cold", staphylococcal abscesses. *Lancet* 1966. **1**: 1013–1015.

11 Hill, H. R., Ochs, H. D., Quie, P. G., Clark, R. A., Pabst, H. F., Klebanoff, S. J. and Wedgwood, R. J., Defect in neutrophil granulocyte chemotaxis in Job's syndrome of recurrent "cold" staphylococcal abscesses. *Lancet* 1974. **2**: 617–619.

12 Donabedian, H. and Gallin, J. I., The hyperimmunoglobulin E recurrent-infection (Job's) syndrome. A review of the NIH experience and the literature. Medicine 1983. **62**: 195–208.

13 Mintz, R., Garty, B. Z., Meshel, T., Marcus, N., Katanov, C., Cohen-Hillel, E. and Ben-Baruch, A., Reduced expression of chemoattractant receptors by polymorphonuclear leukocytes in hyper IgE syndrome patients. *Immunol. Lett* 2010. **130**: 97–106.

14 Wolach, B., Gavrieli, R., Roos, D. and Berger-Achituv, S., Lessons learned from phagocytic function studies in a large cohort of patients with recurrent infections. *J. Clin. Immunol.* 2012. **32**: 454–466.

15 Wolach, O., Kuijpers, T., Ben-Ari, J., Gavrieli, R., Feinstein-Goren, N., Alders, M., Garty, B. Z. et al., Variable clinical expressivity of STAT3 mutation in hyperimmunoglobulin E syndrome: genetic and clinical studies of six patients. *J. Clin. Immunol.* 2014. **34**: 163–170.

16 Fontan, G., Lorente, F., Garcia Rodriguez, M. C. and Ojeda, J. A., Defective neutrophil chemotaxis and hyperimmunoglobulinemia E-a reversible defect? *Acta Paediatr. Scand.* 1976. **65**: 509–511.

17 Mawhinney, H., Killen, M., Fleming, W. A. and Roy, A. D., The hyperimmunoglobulin E syndrome—a neutrophil chemotactic defect reversible by histamine H2 receptor blockade? *Clin. Immunol. Immunopathol.* 1980. **17**: 483–491.

18 Dreskin, S. C. and Gallin, J. I., Evolution of the hyperimmunoglobulin E and recurrent infection (HIE, JOB's) syndrome in a young girl. *J. Allergy Clin. Immunol.* 1987. **80**: 746–751.

19 Hansel, T. T., Pound, J. D. and Thompson, R. A., Isolation of eosinophils from human blood. *J. Immunol. Methods* 1990. **127**: 153–164.

20 Hosoki, K., Nakamura, A., Nagao, M., Hiraguchi, Y., Tanida, H., Tokuda, R., Wada, H. et al., *Staphylococcus aureus* directly activates eosinophils via platelet-activating factor receptor. *J. Leukoc. Biol.* 2012. **92**: 333–341.

21 Prince, L. R., Graham, K. J., Connolly, J., Anwar, S., Ridley, R., Sabroe, I., Foster, S. J. et al., *Staphylococcus aureus* induces eosinophil cell death mediated by alpha-hemolysin. *PLoS One* 2012. **7**: e31506.

22 Pang, Y. Y., Schwartz, J., Thoendel, M., Ackermann, L. W., Horswill, A. R. and Nauseef, W. M., agr-Dependent interactions of *Staphylococcus aureus* USA300 with human polymorphonuclear neutrophils. *J. Innate Immun.* 2010. **2**: 546–559.

23 Morfeldt, E., Panova-Sapundjieva, I., Gustafsson, B. and Arvidson, S., Detection of the response regulator AgrA in the cytosolic fraction of *Staphylococcus aureus* by monoclonal antibodies. *FEMS Microbiol. Lett.* 1996. **143**: 195–201.

24 Zhang, L. Y., Tian, W., Shu, L., Jiang, L. P., Zhan, Y. Z., Liu, W., Zhao, X. D. et al., Clinical features, STAT3 gene mutations and Th17 cell analysis in nine children with hyper-IgE syndrome in mainland China. *Scand. J. Immunol.* 2013. **78**: 258–265.

25 Whyte, M. K., Meagher, L. C., MacDermot, J. and Haslett, C., Impairment of function in aging neutrophils is associated with apoptosis. *J. Immunol.* 1993. **150**: 5124–5134.

26 Epling-Burnette, P. K., Zhong, B., Bai, F., Jiang, K., Bailey, R. D., Garcia, R., Jove, R. et al., Cooperative regulation of Mcl-1 by Janus kinase/stat and phosphatidylinositol 3-kinase contribute to granulocyte-macrophage colony-stimulating factor-delayed apoptosis in human neutrophils. *J. Immunol.* 2001. **166**: 7486–7495.

27 van Raam, B. J., Drewniak, A., Groenewold, V., van den Berg, T. K. and Kuijpers, T. W., Granulocyte colony-stimulating factor delays neutrophil apoptosis by inhibition of calpains upstream of caspase-3. *Blood* 2008. **112**: 2046–2054.

28 Sakamoto, E., Hato, F., Kato, T., Sakamoto, C., Akahori, M., Hino, M. and Kitagawa, S., Type I and type II interferons delay human neutrophil apoptosis via activation of STAT3 and up-regulation of cellular inhibitor of apoptosis 2. *J. Leukoc. Biol.* 2005. **78**: 301–309.

29 Freeman, A. F. and Holland, S. M., Clinical manifestations of hyper IgE syndromes. *Dis. Markers* 2010. **29**: 123–130.

30 Greenlee-Wacker, M. C., Rigby, K. M., Kobayashi, S. D., Porter, A. R., DeLeo, F. R. and Nauseef, W. M., Phagocytosis of *Staphylococcus aureus* by human neutrophils prevents macrophage efferocytosis and induces programmed necrosis. *J. Immunol.* 2014. **192**: 4709–4717.

31 Sastalla, I., Williams, K. W., Anderson, E. D., Myles, I. A., Reckhow, J. D., Espinoza-Moraga, M., Freeman, A. F. et al., Molecular typing of

Staphylococcus aureus isolated from patients with autosomal dominant hyper IgE syndrome. *Pathogens* 2017. **6**: 23.

32 Myles, I. A., Anderson, E. D., Earland, N. J., Zarember, K. A., Sastalla, I., Williams, K. W., Gough, P. et al., TNF overproduction impairs epithelial staphylococcal response in hyper IgE syndrome. *J Clin Invest.* 2018. **128**: 3595–3604.

33 Choi, S. M., McAleer, J. P., Zheng, M., Pociask, D. A., Kaplan, M. H., Qin, S., Reinhart, T. A. et al., Innate Stat3-mediated induction of the antimicrobial protein Reg3gamma is required for host defense against MRSA pneumonia. *J. Exp. Med.* 2013. **210**: 551–561.

34 Steward-Tharp, S. M., Laurence, A., Kanno, Y., Kotlyar, A., Villarino, A. V., Sciume, G., Kuchen, S. et al., A mouse model of HIES reveals pro- and anti-inflammatory functions of STAT3. *Blood* 2014. **123**: 2978–2987.

35 Cederlund, A., Olliver, M., Rekha, R. S., Lindh, M., Lindbom, L., Normark, S., Henriques-Normark, B. et al., Impaired release of antimicrobial peptides into nasal fluid of hyper-IgE and CVID patients. *PLoS One* 2011. **6**: e29316.

36 Kuijpers, T. W., Tool, A. T., van der Schoot, C. E., Ginsel, L. A., Onderwater, J. J., Roos, D. and Verhoeven, A. J., Membrane surface antigen expression on neutrophils: a reappraisal of the use of surface markers for neutrophil activation. *Blood* 1991. **78**: 1105–1111.

37 Roos, D. and de Boer, M., Purification and cryopreservation of phagocytes from human blood. *Methods Enzymol.* 1986. **132**: 225–243.

38 Ide, M., Weiler, D., Kita, H. and Gleich, G. J., Ammonium chloride exposure inhibits cytokine-mediated eosinophil survival. *J. Immunol. Methods* 1994. **168**: 187–196.

39 Malm-Erjefalt, M., Stevens, T. R., Persson, C. G. and Erjefalt, J. S., Discontinuous Percoll gradient centrifugation combined with immunomagnetic separation obviates the need for erythrocyte lysis and yields isolated eosinophils with minimal granule abnormalities. *J. Immunol. Methods* 2004. **288**: 99–109.

40 Ferrante, A. and Thong, Y. H., Optimal conditions for simultaneous purification of mononuclear and polymorphonuclear leucocytes from human blood by the Hypaque-Ficoll method. *J. Immunol. Methods* 1980. **36**: 109–117.

41 Sundin, M., Tesi, B., Sund Bohme, M., Bryceson, Y. T., Putsep, K., Chiang, S. C., Thunberg, S. et al., Novel STAT3 mutation causing hyper-IgE syndrome: studies of the clinical course and immunopathology. *J. Clin. Immunol.* 2014. **34**: 469–477.

42 Kenzel, S., Mergen, M., von Susskind-Schwendi, J., Wennekamp, J., Deshmukh, S. D., Haeffner, M., Triantafyllopoulou, A. et al., Insulin modulates the inflammatory granulocyte response to streptococci via phosphatidylinositol 3-kinase. *J. Immunol.* 2012. **189**: 4582–4591.

Abbreviations: **agr**: accessory gene regulator · **BAL**: bronchoalveolar lavage · **DMSO**: dimethyl sulfoxide · **HIES**: hyper-IgE syndrome · **LDH**: lactate dehydrogenase · **NET**: neutrophil extracellular trap · **PMA**: phorbol 12-myristate 13-acetate · **S. aureus**: *Staphylococcus aureus* · T_H17: T helper 17 cells · **vcl**: vehicle

Full correspondence: Dr. Susan Farmand, Microbial Pathogenesis, M1, Gustav V, Karolinska University Hospital Solna, 17176 Stockholm
e-mail: susan.farmand@ki.se